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2) **The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury.**
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4) **The mitochondrial permeability transition pore and its role in cell death.**
Crompton M
Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K. m.crompton@biochemistry.ucl.ac.uk
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5) **The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins.**
Marzo I; Brenner C; Zamzami N; Susin S A; Beutner G; Brdiczka D; Remy R; Xie Z H; Reed J C; Kroemer G
Centre National de la Recherche Scientifique, Unite Propre de Recherche 420, F-94801 Villejuif, France.
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Department of Biochemistry and Molecular Biology, University College London, UK. m.crompton@bsm.biochemistry.ucl.ac.uk
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The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury

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Abstract

The mitochondrial permeability transition (mPT) involves the opening of a non-specific pore in the inner membrane of mitochondria, converting them from organelles whose production of ATP sustains the cell, to instruments of death. Here, I first summarize the evidence in favour of our model for the molecular mechanism of the mPT. It is proposed that the adenine nucleotide translocase (ANT) is converted into a non-specific pore through a calcium-mediated conformational change. This requires the binding of a unique cyclophilin (cyclophilin-D, CyP-D) to the ANT, except when matrix [Ca^{2+}] is very high. Binding of CyP-D is increased in response to oxidative stress and some thiol reagents which sensitize the mPT to [Ca^{2+}]. Matrix adenine nucleotides decrease the sensitivity of the mPT to [Ca^{2+}] by binding to the ANT. This is antagonized by carboxyatractyloside (an inhibitor of the ANT) and by modification of specific thiol groups on the ANT by oxidative stress or thiol reagents; such treatments thus enhance the mPT. In contrast, decreasing intracellular pH below 7.0 greatly desensitizes the mPT to [Ca^{2+}]. Conditions which sensitize the mPT towards [Ca^{2+}] are found in hearts reperfused after a period of ischaemia, a process that may irreversibly damage the heart (reperfusion injury). We have demonstrated directly that mPT pores open during reperfusion (but not ischaemia) using a technique that involves entrapment of [^{3}H]deoxyglucose in mitochondria that have undergone the mPT. The mPT may subsequently reverse in hearts that recover from ischaemia/reperfusion, the extent of resealing correlating with recovery of heart function. A variety of agents that antagonize the mPT protect the heart from reperfusion injury, including cyclosporin A, pyruvate and propofol. Mitochondria that undergo the mPT and then reseal may cause cytochrome *c* release and thus initiate apoptosis in cells subjected to stresses less severe than those causing necrosis. An example is the apoptotic cell death in the hippocampus that occurs several days

after insulin-induced hypoglycaemia, and can be prevented by prior treatment with cyclosporin A.

Introduction

Essential for normal mitochondrial function is that the inner membrane remains impermeable to all but a few selected metabolites and ions. If this permeability barrier were to break down within the cell, ATP concentrations could not be maintained, even by glycolysis, since the proton-translocating ATPase of the uncoupled mitochondria would actively hydrolyse rather than synthesize ATP. A cell compromised in this way would be destined to die, since ATP is required to maintain its functional integrity and ionic homoeostasis. Eventually, the permeability barrier of the plasma membrane would be breached through phospholipase A_2 action, and total disruption of cellular metabolite and ion concentrations would make cell death inevitable. Such a non-specific increase in the permeability of the inner mitochondrial membrane (IMM) can occur through a process known as the mitochondrial permeability transition (mPT). This process occurs when the mitochondrial matrix [Ca^{2+}] is greatly increased, especially when this is accompanied by oxidative stress, adenine nucleotide depletion and mitochondrial depolarization. It is caused by the opening of a non-specific pore in the IMM, which transports any molecule of <1500 Da [1–4]. The conditions required to induce the mPT are similar to those experienced by tissues such as the heart when they are reperfused following an extended (>20 min) period of ischaemia [2,4]. It is well known that such reperfusion exacerbates the damage caused by ischaemia itself. Indeed, this reperfusion injury is a major problem associated with open heart surgery where the heart must be stopped during the surgical procedure and then restarted again. In this article I will give a brief review of what is known about the molecular mechanism of the mPT and its regulation. I will then summarize the evidence for a critical role of the mPT in reperfusion injury and how our knowledge of the mechanism of the mPT can provide insights into how the heart might be protected from reperfusion injury.

The molecular mechanism of the mPT

Early data showed that elevated matrix [Ca^{2+}] is required to trigger the mPT, but that the sensitivity to [Ca^{2+}] could be greatly increased by a variety of 'inducers' such as oxidative stress, phosphate, the inhibitor of the adenine nucleotide translocase (ANT) carboxyatractyloside (CAT) and depletion of matrix adenine nucleotides. Sensitivity to [Ca^{2+}] is reduced by other factors such as ADP, low pH and bongkrekic acid (BKA) [4–7]. All solutes <1500 Da enter the mitochondria following the mPT and the process is reversed immediately by chelation of Ca^{2+} with EGTA [6–9]. Although these data clearly imply the presence of a Ca^{2+}-dependent, non-selective channel in the IMM, for many years it was argued that a phospholipase A_2-mediated increase in the permeability of the inner membrane phospholipid bilayer was responsible [10]. The

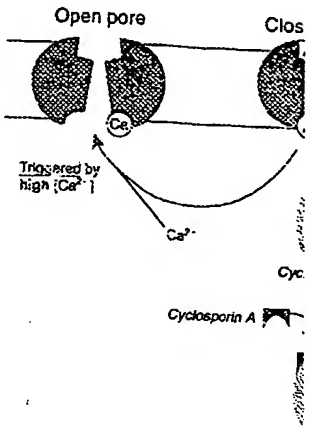

Figure 1 Proposed model for the the absence of CyP-D binding to the [Ca^{2+}], but binding of CyP-D enables occur at lower [Ca^{2+}]. CyP binding reagents, chaotropic agents and increa the mPT to [Ca^{2+}]. In contrast, adenir face of the ANT, which is enhanced by desensitizes the mPT to [Ca^{2+}]. Adeni 'c' conformation of ANT, thiol reagent the mPT to occur at higher $\Delta\Psi$ and lo probably inhibit pore opening by comp site.

one observation more than any other t onstration by Crompton and colleagu the mPT can be specifically and totally tions of cyclosporin A (CsA) without : [11–13]. CsA is a drug with powerful in ated through an inhibition of calc phosphatase, by a complex formed b (CyP), CyP-A [14]. This suggested to u also involve a member of the CyP fam studies in this and other laboratories ha led us to propose a mechanism for the involves the formation of a non-specific formational change of the ANT. It is su Ca^{2+} and facilitated by the binding of a tein with peptidylprolyl *cis–trans* isome model and the mechanisms by which fa act are presented later and are summariz

nd can be prevented by prior treatment

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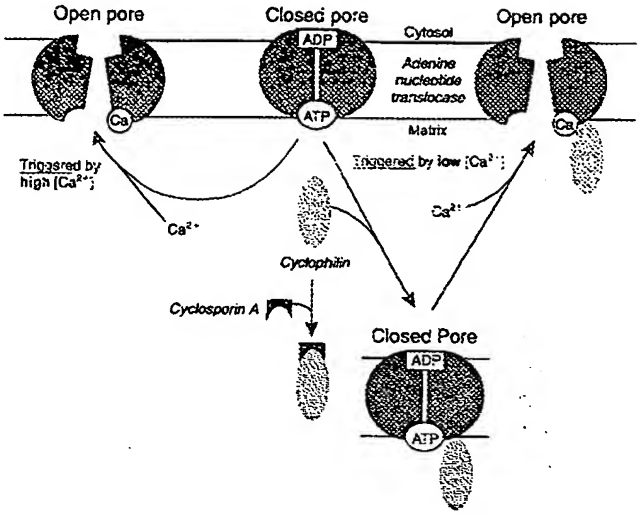

Figure 1 Proposed model for the mechanism of action of the mPT. In the absence of CyP-D binding to the ANT, the mPT can still open at high [Ca^{2+}], but binding of CyP-D enables the required conformational change to occur at lower [Ca^{2+}]. CyP binding is increased by oxidative stress, thiol reagents, chaotropic agents and increased matrix volume, which all sensitize the mPT to [Ca^{2+}]. In contrast, adenine-nucleotide binding to the matrix surface of the ANT, which is enhanced by the 'm' conformation of ANT and $\Delta\Psi$, desensitizes the mPT to [Ca^{2+}]. Adenine-nucleotide binding is inhibited by the 'c' conformation of ANT, thiol reagents and oxidative stress, which thus allow the mPT to occur at higher $\Delta\Psi$ and lower [Ca^{2+}]. [Mg^{2+}] and [H^+] (low pH) probably inhibit pore opening by competing directly with Ca^{2+} for the trigger site.

one observation more than any other that overturned this view was the demonstration by Crompton and colleagues, and subsequently many others, that the mPT can be specifically and totally inhibited by submicromolar concentrations of cyclosporin A (CsA) without any effect on phospholipase A_2 activity [11–13]. CsA is a drug with powerful immunosuppressive activity that is mediated through an inhibition of calcineurin, a calcium-sensitive protein phosphatase, by a complex formed between CsA and cytosolic cyclophilin (CyP), CyP-A [14]. This suggested to us that effects of CsA on the mPT might also involve a member of the CyP family within the matrix [13]. Subsequent studies in this and other laboratories have confirmed this suggestion and have led us to propose a mechanism for the mPT, shown in Figure 1. Our model involves the formation of a non-specific channel occurring as a result of a conformational change of the ANT. It is suggested that this process is triggered by Ca^{2+} and facilitated by the binding of another matrix protein, CyP-D, a protein with peptidylprolyl *cis–trans* isomerase (PPIase) activity. Evidence for this model and the mechanisms by which factors known to regulate the mPT may act are presented later and are summarized in Tables 1 and 2 respectively.

Table 1 Summary of the evidence for a role for the ANT and CyP in the formation of the PT pore

Details are presented in the text. TBH, t-butylhydroperoxide.

CyP involvement

- Mitochondria contain a unique nuclear-encoded CyP, CyP-D, with peptidyl-prolyl *cis–trans* isomerase PPIase activity that is inhibited by CsA
- The K_i for inhibition of this PPIase activity by a range of CsA analogues matches their $K_{0.5}$ values for inhibition of the mPT
- CyP-D binds in a CsA-sensitive manner to the IMM. Binding is increased by oxidative stress, thiol reagents, chaotropic agents and increased matrix volume that also stimulate the mPT

ANT involvement

- The mPT is sensitized to $[Ca^{2+}]$ by CAT, which locks the ANT in the 'c' conformation, and is inhibited by BKA, which locks the ANT in the 'm' conformation
- The mPT is inhibited by micromolar ADP concentrations in the matrix, and this effect is antagonized by CAT. Only nucleotides that bind to the ANT inhibit the mPT
- Oxidative stress (TBH) and thiol reagents (diamide, phenylarsine oxide, eosine maleimide) stimulate the mPT and reduce the effectiveness of ADP as an inhibitor of the mPT by two orders of magnitude or more
- The ANT is known to have thiol groups that are reactive towards oxidative stress and phenylarsine oxide. Eosine maleimide specifically modifies Cys^{159} of the ANT
- The ANT is an electrogenic carrier and is thus a suitable candidate for the membrane potential sensor of the mPT. Opening of the mPT in adenine nucleotide-depleted mitochondria is insensitive to the membrane potential
- The purified and reconstituted ANT can form a non-selective channel at high $[Ca^{2+}]$ or when specific thiol groups are modified. Although similar to the mPT pore, it is insensitive to CsA

An ANT–CyP-D complex

- The purified ANT and that of solubilized IMMs binds tightly and in a CsA-inhibitable manner to immobilized GST–CyP-D
- The purified ANT, reconstituted in the presence of the GST–CyP-D, can form a non-selective channel at low $[Ca^{2+}]$ that is inhibited by CsA

Techniques for measuring the mPT in isolated mitochondria

All investigations on the mechanism of the mPT require the use of a reliable assay method and several are in common use. The most direct is the sucrose-entrapment technique introduced by Crompton and co-workers in which permeation of [^{14}C]sucrose (or other solutes) into the matrix is determined [8,9,15,16]. Using rapid reaction techniques this method has been used to characterize the kinetics and specificity of the pore, but is relatively cumbersome and does not allow continuous monitoring of the process. The most commonly used continuous assay utilizes the decrease in light scattering that occurs as mitochondria swell during the mPT [5–7,13]. Swelling takes place as small-molecular-mass solutes equilibrate through the non-specific pore while higher-molecular-mass solutes continue to exert an osmotic pressure which

Table 2 Proposed sites of act

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Oxidative stress (e.g. TBH)	Oxid (e.g.
Increased matrix volume	'c' c
Chaotropic agents	
Inhibitory	
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Table 2 Proposed sites of action of known modulators of the mPT

Note that both CyP and ADP binding exert their effects through changes in the sensitivity of the mPT to $[Ca^{2+}]$. The locus of action of arginine-specific reagents such as phenylglyoxal has not been defined but these agents appear to inhibit the mPT induced by a wide range of effectors. PheArs, phenylarsine oxide; TBH, t-butylhydroperoxide.

Effect via change in CyP-D binding	Effect via change in nucleotide binding	Direct effect on Ca^{2+} binding
Activatory		
Thiol reagents (e.g. diamide, PheArs)	Thiol reagents (e.g. diamide, PheArs)	High pH
Oxidative stress (e.g. TBH)	Oxidative stress (e.g. TBH)	
Increased matrix volume	'c' conformation of ANT	
Chaotropic agents		
Inhibitory		
CsA	Membrane potential	Low pH
	Membrane surface charge (e.g. trifluoperazine)	Mg^{2+}
	'm' conformation of ANT	Arginine reagents
	Arginine reagents	

drives water into the matrix. A modification of this assay is to fully open the mPT pore by exposure of mitochondria to high $[Ca^{2+}]$ and then measure mitochondrial shrinkage that occurs upon addition of poly(ethylene glycol) that is too large to enter the matrix through the pore [7,17–19]. The advantage of this technique is that it allows the composition of the mitochondrial matrix to be modified when effectors of the mPT working on the matrix surface are to be studied. Another continuous assay that has been employed follows the decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) or release of accumulated matrix Ca^{2+} caused by the massive increase in permeability to protons and Ca^{2+} that occurs during the mPT [3,10]. This can be determined using ion-sensitive electrodes and dyes, but suffers from the disadvantage that any uncoupling of the mitochondria by other causes will produce a similar result. Discrimination is achieved through the use of CsA, but this is not without its problems. There is now evidence that there may be substates of the mPT pore that transport H^+ and Ca^{2+} but not larger solutes [20,21], and it is also known that the mPT becomes CsA-insensitive when $[Ca^{2+}]$ is high [18,19,22–24].

A major problem of many published studies on the regulation and mechanism of the mPT is that the methods used do not allow discrimination between factors directly affecting the mPT mechanism and those working indirectly through changes in other parameters such as matrix $[Ca^{2+}]$ and membrane potential. In our own laboratory we have endeavoured to simplify the number of variables that may contribute to the regulation of the mPT and have usually used de-energized mitochondria, more recently in the presence of

the calcium ionophore A23187 [13,17–19,24]. This ensures that $\Delta\Psi_m$ and matrix $[Ca^{2+}]$ are held constant, and thus factors influencing the mPT cannot be doing so indirectly through these parameters. We have also used a very sensitive continuous assay of light scattering to determine mitochondrial swelling and shrinkage, which has allowed the determination of mPT kinetics [13,19,24].

The role of mitochondrial CyP in the mPT

We and others have demonstrated that the mitochondrial matrix contains a PPIase that is inhibited by CsA and its analogues with $K_{0.5}$ values very similar to those for inhibition of the mPT [13,24–27]. Furthermore, the number of binding sites required for 100% inhibition of the mPT corresponds to the concentration of PPIase within the matrix (about 50 pmol per mg of protein) [13,25]. Purification and N-terminal sequencing of this PPIase confirmed that it was a member of the CyP family, most probably identical to the product of the human CyP-3 gene [28,29]. This nuclear-encoded protein is now more usually termed CyP-D and has a mitochondrial targeting presequence that is cleaved after translocation of the protein into the matrix. We have cloned and sequenced the cDNA for rat mitochondrial CyP (accession number U68544) and, with the exception of the extreme N-terminal residue, the sequence corresponds exactly to the N-terminus sequence of the purified protein [27,28]. This confirms that the matrix PPIase in rat mitochondria is indeed the equivalent of human CyP-3. Removal of the mitochondrial targeting presequence from the CyP-D takes place in the matrix and may occur at one of two points leading to mature proteins of about 17.6 kDa (minor product) and 18.6 kDa (major product) [27,28]. Crompton and colleagues have also purified and sequenced a mitochondrial CyP that is associated with the IMM and which proved to be CyP-D [30,31]. We have used Northern blotting to show that mRNA for CyP-D in rat muscle, heart, liver, kidney and brain is of identical size (1.5 kb), making it unlikely that there are differently spliced tissue-specific isoforms [28].

A wide range of inducers can sensitize the mPT to $[Ca^{2+}]$, and our model suggests that one way this might be achieved is by enhancing CyP-D binding to the membrane component(s) of the mPT pore. To investigate this possibility we developed a method to measure the amount of CyP-D bound to IMMs. This involved the rapid isolation of IMMs following treatment of mitochondria with inducers that enhance the sensitivity of the mPT to $[Ca^{2+}]$, followed by SDS/PAGE and Western blotting with anti-CyP-D antibodies [17,18]. Oxidative stress induced with t-butylhydroperoxide (TBH), glutathione depletion induced by diamide treatment or modification of vicinal thiols by phenylarsine oxide (PheAs) were all shown to increase CyP binding to the IMM concomitant with their ability to increase the sensitivity of pore opening to $[Ca^{2+}]$ [17–19]. Mild chaotropic agents such as KSCN and increases in matrix volume were also able to increase CyP binding in parallel with their ability to sensitize the mPT to $[Ca^{2+}]$ [18]. In all cases, binding of CyP was almost totally prevented by CsA. In contrast, we found that several other modulators of the mPT, such as matrix $[Ca^{2+}]$, [ADP], pH or membrane potential

were without effect on CyP bindi
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The role of the ANT in the mPT

The ANT was first implicate
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were without effect on CyP binding [18,19], although other workers, using different techniques, have obtained conflicting results [30–32].

Although the evidence for a role of CyP-D in the mPT presented earlier is strong, there is a body of data that imply that CyP-D binding may not be essential for the opening of the mPT pore. Rather, it seems that the role of CyP-D may be to facilitate a conformational change of the ANT that otherwise would only occur at very high $[Ca^{2+}]$. Thus, at high matrix $[Ca^{2+}]$, inhibition of pore opening by CsA is overcome in both heart and liver mitochondria [18,19,22–24]. Yet under the same conditions, CsA is able to prevent almost totally the binding of CyP-D to the IMM [17,19]. Furthermore, the purified ANT is capable of behaving as a non-specific pore at high (millimolar) $[Ca^{2+}]$ as will be described later.

The role of the ANT in the mPT

The ANT was first implicated in the mPT when it was observed that any reagent, such as CAT, that stabilized the 'c' (cytoplasmic) conformation of the ANT, stimulated the mPT, whereas BKA, which stabilized the 'm' (matrix) conformation of the ANT, inhibited the mPT [13,19,33]. Furthermore, matrix ADP is an important modulator of pore opening that acts by decreasing the sensitivity of the calcium trigger site to $[Ca^{2+}]$ [6,19]. There are two ADP binding sites with K_i values of about 1 and 25 μM, the former being blocked by CAT [19,22,34]. We have tested the ability of a range of nucleotides to inhibit the mPT, and found that only ATP and deoxy-ADP inhibit, their $K_{0.5}$ values being, respectively, 500 and 20 times greater than ADP. This correlates with their affinity for the matrix-binding site of the ANT [19]. Adenine nucleotide binding is antagonized by oxidative stress induced by TBH or diamide and also by thiol reagents such as PheArs and eosine maleimide, which are known to be powerful activators of the mPT [19]. PheArs has the greatest effect of the reagents tested, raising the $K_{0.5}$ for ADP inhibition of the mPT to >500 μM; this is accompanied by covalent modification of the ANT [18]. Furthermore, eosine maleimide is known to attack Cys^{159} of the ANT and inhibit activity of the translocase by preventing ADP binding to the matrix-binding site [35,36].

Bernardi and colleagues have provided strong evidence for regulation of the mPT by the $\Delta\Psi_m$, activation occurring as $\Delta\Psi_m$ becomes less negative [37–40]. We have demonstrated that in mitochondria depleted of adenine nucleotides by pyrophosphate treatment, not only is the mPT much more sensitive to $[Ca^{2+}]$, but it is also no longer sensitive to $\Delta\Psi_m$ [19,25]. Thus, the regulation of the mPT by $\Delta\Psi_m$ is dependent on the presence of matrix adenine nucleotides and may involve the ANT itself responding to $\Delta\Psi_m$ through an effect on adenine nucleotide binding. The ANT is an electrogenic carrier, transporting ATP^{4-} in exchange for ADP^{3-}. Its mechanism may well involve a potential-driven conformational change that alters the affinity of the adenine nucleotides on either side of the membrane [41–43]. A large negative $\Delta\Psi_m$ will enhance ATP binding to the ANT and thus inhibit the mPT. Oxidative stress shifts the voltage dependence of the mPT, allowing the pore to open at more negative potentials. Two distinct thiol groups have been implicated in this effect, one sensitive to oxidation of glutathione, for example by TBH or

diamide, and the other responding to the redox state of matrix NAD(P) [39,44,45]. The ANT is known to have three cysteine residues that show differential reactivity to various thiol reagents in a conformation-dependent manner [35,36]. These are Cys^{56}, Cys^{159} and Cys^{256} and, as discussed earlier, it seems likely that modification of Cys^{159} is responsible for the ability of oxidative stress and thiol reagents to reduce the inhibitory effects of ADP and membrane potential on the mPT [19]. Modification of Cys^{56} is probably responsible for the effects of oxidative stress and thiol reagents on CyP-D binding to the ANT [19]. Bernardi and colleagues have recently demonstrated that the arginine-specific reagents phenylglyoxal and 2,3-butanedione strongly favour the closed state of the mPT pore, even in the presence of strong inducers such as high $[Ca^{2+}]$ and ADP depletion [46,47]. The location of the arginine groups responsible for this effect has not been established but they are accessible from the matrix surface and the ANT has a large number of arginines on the three matrix-facing loops.

Although a role for the ANT in the mPT is now generally accepted (see [1–3]), there has been debate as to whether this protein may itself form the pore or rather be a regulatory component. In particular, there was no direct evidence for an interaction of CyP-D with the ANT. Our own attempts at chemical cross-linking of the CyP-D to the ANT have so far met with no success. As an alternative strategy we have overexpressed CyP-D as a glutathione S-transferase (GST) fusion protein and used this to investigate what protein(s) of the IMMs bind to it [4,48]. We first demonstrated that the purified GST–CyP-D fusion protein could bind to IMMs in a CsA-sensitive manner and that this binding was enhanced by diamide treatment. We then solubilized the IMMs in Triton X-100 and incubated these with GST–CyP-D immobilized on Sepharose. Bound proteins were washed extensively and then eluted with glutathione and analysed by SDS/PAGE and Western blotting with anti-ANT and porin antibodies. A major protein band of 30 kDa was found to bind in a CsA-sensitive manner and this correlated with the presence of a strong ANT-reactive band on the Western blot. Binding of the ANT was prevented by both CAT and BKA, suggesting that it depended on the ANT undergoing conformational change. No porin binding was observed under the same conditions [4,48].

Other possible components of the mPT pore

The evidence presented earlier confirms an interaction of CyP-D with the ANT, but this does not allow us to conclude that the ANT and CyP-D alone are sufficient to form the mPT pore. It has been reported that just the purified and reconstituted ANT alone can form Ca^{2+}-dependent channels resembling the PT pore [49,50], but the opening of these channels required concentrations of $[Ca^{2+}]$ of more than 0.5 mM and was not CsA-sensitive. However, we have recently reconstituted the purified ANT into proteoliposomes in the presence of GST–CyP-D and measured Ca^{2+}-dependent pore opening by the release of entrapped malate (A. Rück, J. Gillespie, D. Brdiczka and A. Halestrap, unpublished work). Whereas in the absence of GST–CyP-D the purified ANT could be induced to form a pore only at $[Ca^{2+}]>0.25$ mM [50], in its presence only

5–10 μM Ca^{2+} was required [48]. Fu
GST–CyP-D became sensitive to i
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5–10 μM Ca^{2+} was required [48]. Furthermore, pore opening in the presence of GST–CyP-D became sensitive to inhibition by CsA, which it was not in its absence [48]. Thus it does appear that the minimum requirement for the mPT pore formation is the presence of ANT and CyP-D, as we originally proposed [13], although this does not exclude the possibility of other components acting in a regulatory manner. A particularly interesting question that needs to be resolved is where the calcium trigger site is located. There are no obvious Ca^{2+}-binding motifs on either the ANT or CyP-D, yet calcium-dependent pore opening occurs in the ANT–CyP-D complex without the need for additional calcium-binding proteins. We have demonstrated that matrix calcium at micromolar concentrations or less can induce a conformational change in the ANT [13,51] and thus it seems most likely that there is a calcium-binding site associated with the matrix loops of this protein. There are several conserved aspartates and glutamates in the three matrix loops and these may orientate in such a way as to produce a calcium-binding motif.

Those advocating a central role of the mPT in apoptosis often state that the mPT pore complex includes the outer membrane components, porin and the benzodiazepine receptor (see for example [52,53]). Whereas there are reports that these components may co-purify with the ANT as a complex under some conditions [54,55], our own studies provide no support for their direct involvement in the pore complex [19,48]. Furthermore, we have presented evidence that argues against their playing a role in regulation of the mPT. First, ligands of the mitochondrial benzodiazepine receptor are without any direct effect on the mPT [19]. Second, the mPT can be observed in mitoplasts from which the outer membrane has been largely removed by digitonin treatment and exhibits identical properties to the mPT in normal mitochondria [19].

The locus of action of different modulators of the mPT

In Table 2 I summarize how the ANT may provide a common locus for the action of the major modulators of the mPT. Binding of matrix adenine nucleotides to the ANT inhibits pore formation by decreasing the affinity of the trigger site for $[Ca^{2+}]$. Adenine nucleotide binding is antagonized by the 'c' conformation of the carrier, membrane depolarization and thiol reagents that attack Cys^{159} of the ANT. These all sensitize the mPT to $[Ca^{2+}]$. In contrast, the 'm' conformation and increased membrane potential enhance nucleotide binding and inhibit the pore. These effectors are all without effect on CyP-D recruitment to the membrane. Chaotropic agents and increased matrix volume sensitize the mPT to $[Ca^{2+}]$ by increasing CyP-D binding, an effect that thiol reagents and oxidative stress also induce, perhaps through modification of Cys^{56} of the ANT [18]. This facilitates the transition of the ANT into its open channel state. Low pH (<7.0) and increased $[Mg^{2+}]$ are potent inhibitors of the mPT [7,18,19,56,57] and appear to act by direct competition with Ca^{2+} for its binding at the trigger site. Bernardi and colleagues have presented data to show that the effect of low pH involves a specific histidine residue and that this may modulate CyP binding to the IMM [26,32]. However, in our experiments we did not detect such an effect of low pH on CyP binding to either inner membranes [18] or to the purified ANT [48]. Trifluoperazine is a potent inhibitor of

the mPT under energized but not de-energized conditions [19]. It was originally thought to act indirectly through inhibition of phospholipase A_2, preventing the accumulation of free fatty acids which stimulate the mPT, probably through interaction with the ANT [10,58]. However, inhibition occurs even without changes in free fatty acid accumulation and is now thought to be mediated by an effect on surface membrane charge that changes the voltage sensitivity of the mPT [59]. Very recently it has been suggested that in muscle mitochondria the rate of electron flow through complex 1 of the respiratory chain may exert a regulatory effect on the mPT, but the mechanism by which this is mediated remains unclear [60].

The role of the mPT in reperfusion injury of the heart

When ischaemic tissues are reperfused, the damage caused during anoxia is further exacerbated. This phenomenon, known as reperfusion injury, has been widely studied (see [2,4,61–64]) and the mechanisms thought to be involved are summarized in Figure 2. During the ischaemic phase, cells endeavour, unsuccessfully, to maintain their ATP levels through glycolysis, which leads to an accumulation of lactic acid and a decrease in intracellular pH (pH_i) [65]. The Na^+/H^+ antiporter is activated in an attempt to restore pH_i [66,67], but this loads the cell with Na^+, which cannot be pumped out again through the operation of the Na^+/K^+-ATPase if there is insufficient ATP to drive the process [68]. The accumulated Na^+ prevents Ca^{2+} from being pumped out of the cell on the Na^+/Ca^{2+} antiporter and may actually reverse the process, allowing additional Ca^{2+} to enter the cytosol from the plasma [68,69]. Some of this calcium may also enter the mitochondria by reversal of the Na^+/Ca^{2+} antiporter [70], but upon reperfusion Ca^{2+} is rapidly taken up into the mitochondria by means of the uniporter, loading the mitochondrial matrix with large amounts of Ca^{2+} [71,72]. Alone, this might not be sufficient to activate the mPT, but other factors also come into play during reperfusion. The sudden influx of oxygen into the anoxic cell induces the formation of oxygen free radicals through an interaction of oxygen with ubisemiquinone. This accumulates during anoxia as a result of respiratory chain inhibition [2,61,73,74]. Additional oxygen free radicals may be produced through the operation of xanthine oxidase [75]. This enzyme is activated during hypoxia and is presented with high concentrations of xanthine produced by the purine degradation that occurs during ischaemia [76,76a]. The combination of oxidative stress and high $[Ca^{2+}]$ provides the ideal conditions for the mPT, especially in the presence of elevated cellular phosphate concentrations and depleted adenine nucleotide levels, both of which occur during the ischaemic phase [2,61,68]. Furthermore, during the reperfusion phase the pH_i rapidly returns to pre-ischaemic values through the operation of the Na^+/H^+ antiporter, lactic acid efflux on the monocarboxylate transporter and bicarbonate-dependent mechanisms [67]. It will be recalled that low pH (<7.0) exerts a powerful inhibitory effect on the mPT [7,56,57], and when this is removed on reperfusion, the factors that are in place to stimulate the mPT can now exert their full effect.

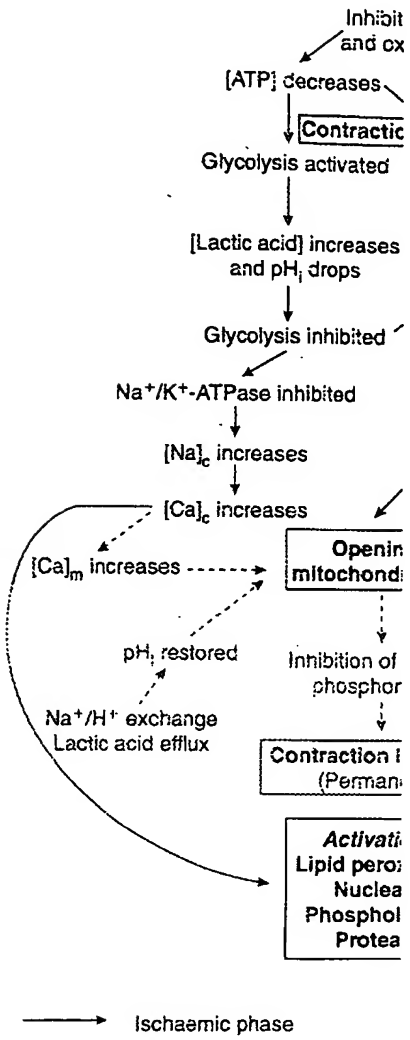

Figure 2 Scheme summarizi reperfusion injury. $[Ca]_m$, matrix and $[Ca^{2+}]$, respectively.

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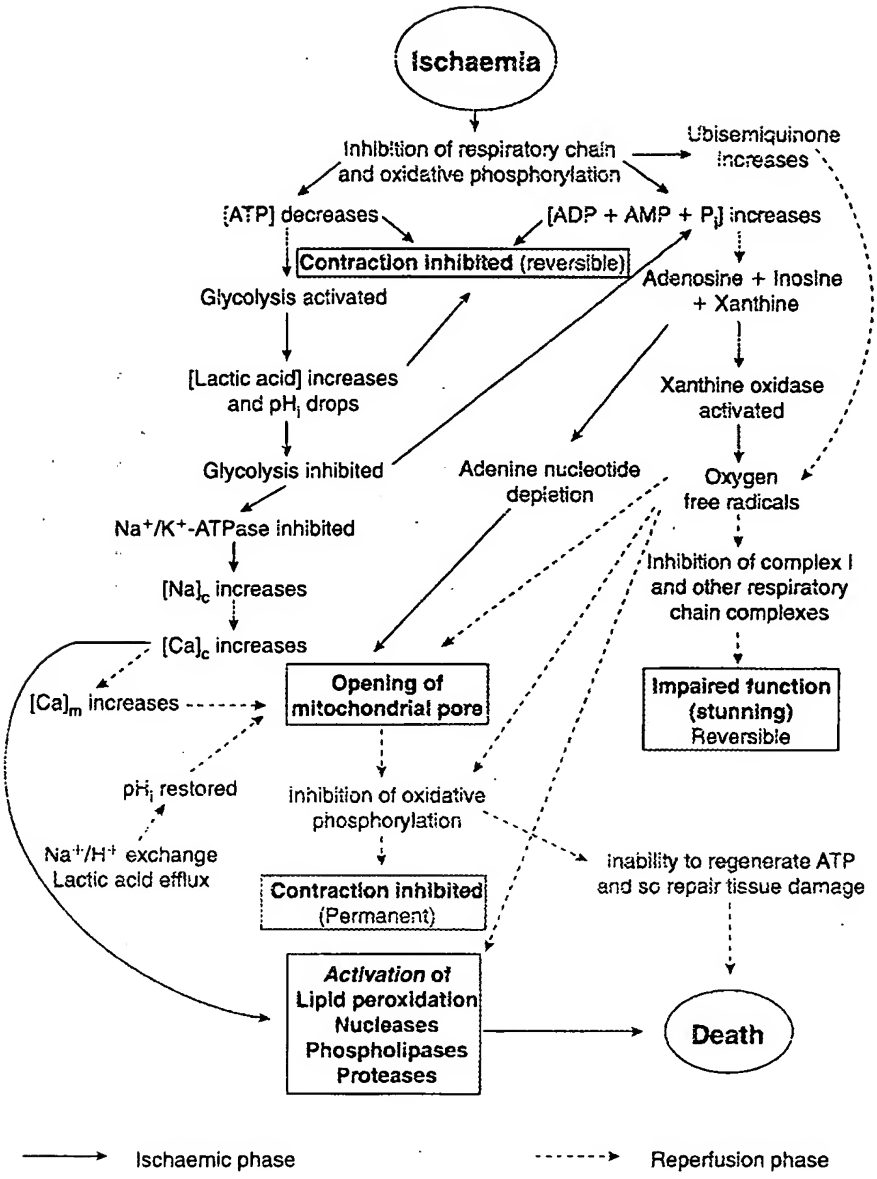

Figure 2 Scheme summarizing the main events that occur in reperfusion injury. $[Ca]_m$, matrix $[Ca^{2+}]$; $[Na]_c$ and $[Ca]_c$, cytoplasmic $[Na^+]$ and $[Ca^{2+}]$, respectively.

The sequence of events described earlier suggests that the mPT may be central in determining whether a cell lives or dies in response to ischaemia and reperfusion. Indeed, morphological studies confirm mitochondria become swollen and amorphous under such conditions [2,62]. If the mPT is a critical

factor in the development of reperfusion injury, CsA would be expected to provide some protection from damage. This has been observed in isolated cardiac myocytes and hepatocytes subjected to re-oxygenation following a period of hypoxia [77,78], in hepatocytes during chemical anoxia and oxidative stress [79–81], in the brain following a hypoglycaemic insult [82] and in perfused livers and hearts subjected to isothermic global ischaemia followed by reperfusion [24,64,83–85]. In the latter case we demonstrated that hearts treated with 0.2 μM CsA showed a greater recovery of left ventricular developed pressure (LVDP), tissue ATP/ADP ratios and functional mitochondria, whereas AMP levels and end-diastolic pressure (an indicator of contracture due to low ATP/ADP and elevated $[Ca^{2+}]$) were lower. No protective effect of CsA was observed on the loss of total adenine nucleotides that occurs as a result of purine degradation during hypoxia. Nor was protection from inhibition of respiratory chain function (state-3 substrate oxidation) observed [24,64,83]. The latter effect is probably caused by oxygen free radicals, formed during ischaemia and reperfusion, directly modifying components of the respiratory chain [24,61,64,83]. Our observations are consistent with CsA exerting its effects by inhibiting the mPT, which is downstream of changes in total adenine nucleotides and free radicals. Furthermore, we have demonstrated that only analogues of CsA that block the mPT in isolated mitochondria are able to offer protection to the reperfused heart [24,64]. No protection is exerted by cyclosporin H and FK506 (another drug whose immunosuppressive action, like that of CsA, is exerted through inhibition of calcineurin), neither of which bind to CyP-D [24,83]. The protective effect of CsA was highly concentration dependent, showing an optimal response at 0.2 μM and declining at higher concentrations [83]. A similar concentration dependence has been observed for CsA protection of isolated cardiac myocytes subjected to re-oxygenation following a period of hypoxia [77].

Direct measurement of pore opening during reperfusion of the ischaemic heart

To establish directly that reperfusion injury of the heart is associated with the mPT it is necessary to measure mitochondrial pore opening *in situ*. Techniques involving the use of fluorescent probes that can easily be applied to isolated cells [80,86] are not appropriate for the perfused heart and we have devised an alternative procedure that relies on the impermeability of the IMM to 2-deoxyglucose 6-phosphate [24,64]. Hearts are perfused in Langendorff recirculating mode with [^{3}H]2-deoxyglucose (DOG), which enters the heart on the glucose carrier and is then phosphorylated to DOG 6-phosphate without further metabolism. Thus [^{3}H]DOG 6-phosphate is trapped in the cytosol of the heart cell, and only enters the mitochondria if the pore opens. Extracellular [^{3}H]DOG is removed form the heart by perfusion in the absence of [^{3}H]DOG, before hearts are subjected to various periods of ischaemia and reperfusion. Mitochondria are then prepared rapidly and assayed for [^{3}H]DOG and citrate synthase (an indicator of mitochondrial recovery). From the [^{3}H]DOG content of the mitochondria and a small sample of total heart homogenate an estimation of pore opening is possible. This technique allowed

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us to demonstrate that mitochondria prepared immediately after the ischaemic period had not taken up DOG 6-phosphate whereas those prepared following a period of reperfusion showed a significant uptake, indicative of pore opening [24]. Maximal mitochondrial DOG uptake was found after 5 min of reperfusion [64], the same time period over which the intracellular pH returns to pre-ischaemic values [67,87]. Thus our data suggest that a profound opening of the mitochondrial pore occurs only during the reperfusion phase, consistent with the predictions made earlier. It has been argued that pore opening is not a primary cause of cell injury but rather a secondary phenomenon that occurs following other critical damage to the myocyte, such as breakdown of the plasma membrane permeability barrier [88]. However, if the latter were to occur, DOG would be lost from the cell before it could enter the mitochondria and thus no increase in mitochondrial DOG would be measured.

Reversal of the mPT in hearts that recover during reperfusion

The extent of functional recovery of the heart during reperfusion can be quantified by using pressure transducers to monitor beat, LVDP, end-diastolic pressure and aortic pressure. In addition, adenine nucleotide concentrations can be determined in hearts freeze–clamped after the ischaemic period or after reperfusion [83]. Reperfusion after short periods of ischaemia leads to total recovery of LVDP and ATP/ADP ratio [83], yet an increase in mitochondrial DOG entrapment is still observed, indicating that the mPT must have occurred even though the heart recovers fully [24,64,83]. This suggests that the mitochondrial pores open in the early phase of reperfusion but then rapidly reseal, allowing total recovery of mitochondrial function and heart performance. Unfortunately, when the mPT reverses, closure of the mitochondrial pores means that DOG remains trapped inside the mitochondria and the reversal is not detected using our normal protocol. However, the DOG technique can be modified to determine whether resealing has occurred. For this purpose, hearts are loaded with [^{3}H]DOG after maximum recovery of the heart has been established during reperfusion. If mitochondrial pores close during reperfusion, DOG entrapment determined using this 'post-loading' protocol should be less than when DOG is present at the start of reperfusion (pre-loading). We have confirmed that this is the case [4,87]. After 40 min ischaemia post-loading gives about 50% less mitochondrial DOG entrapment than observed with pre-loading [4,87]. Thus it would seem that if the insult caused by ischaemia/reperfusion is not too great, mitochondria can undergo a transient PT, followed by closure of the pores and entrapment of the DOG. The closure is presumably brought about by the decrease in matrix [Ca^{2+}] that occurs as calcium is lost from the mitochondria during the mPT, and the removal of oxygen free radicals by superoxide dismutase and glutathione peroxidase. However, this will only occur if enough 'healthy' mitochondria are remaining in the cell to accumulate the released calcium and provide sufficient ATP and NADPH to maintain the ionic and reduced glutathione homoeostasis of the cell. The balance between the number of 'closed' and 'open' mitochondria within any cell will be critical in determining whether a cell lives or dies. If there are too many 'open' mitochondria, they will release more calcium and hydrolyse more ATP

than the 'closed' mitochondria can compensate for. In contrast, if there are sufficient 'closed' mitochondria to meet the cell's ATP requirements and to accumulate released calcium without undergoing the mPT themselves, the 'open' mitochondria will close again and the cell will recover.

Strategies for inhibiting the mPT that can be used to protect the heart from reperfusion injury

Although we have demonstrated that CsA can be used effectively to protect the perfused heart from reperfusion injury, the degree of protection was very sensitive to the concentration of CsA used and to the conditions used for the perfusion ([24,64,83] and P.M. Kerr and A.P. Halestrap, unpublished work). In some situations CsA was actually found to impair recovery of the heart from ischaemia and thus it is unlikely to be appropriate for use in open heart surgery. Consequently, we have investigated the use of other means to inhibit the mPT during reperfusion.

Anti-oxidants and calcium antagonists

There is an extensive literature to show that anti-oxidants and free-radical scavengers can protect the ischaemic/reperfused heart from irreversible damage (see [61,88]). Whereas there are many processes within the cardiac myocyte that are possible targets for the action of these reagents, prevention of the mPT is clearly one of them. Evidence is also strong that protection from reperfusion injury can be achieved by preventing the mitochondrial calcium overload accompanying reperfusion through the use of calcium antagonists or Ruthenium Red, an inhibitor of mitochondrial calcium uptake [71,89–97]. These data are consistent with inhibition of the mPT that such reagents would be predicted to cause.

Low pH_i

It is well established that low pH_i (<7.0), induced by the use of low extracellular pH or by addition of specific inhibitors of the Na^+/H^+ antiporter such as amiloride, can protect a variety of cells, including cardiac myocytes and hepatocytes, from oxidative stress, re-oxygenation following anoxia or reperfusion following ischaemia [78,98–106]. Although low pH_i may have several means of exerting its protective effects, the profound inhibition of the mPT at pH<7.0 is likely to be an important one and has been demonstrated directly in hepatocytes [78]. Furthermore, in the ischaemic/reperfused heart, mitochondrial pores open over the same period of time as the pH_i takes to be restored from <6.5 to pre-ischaemic values (>7.0) [64,67,87].

Pyruvate

It is well documented that pyruvate can protect hearts [107–109] and other tissues [110–112] from ischaemia/reperfusion and anoxia/re-oxygenation injury. The protective effects of pyruvate have been attributed to beneficial metabolic alterations and to protection from free-radical production, since pyruvate acts as a free-radical scavenger [109,110]. However, an additional effect of pyruvate might be through inhibition of the mPT, in part through its

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Propofol

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a free-radical scavenger [121,122] a
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be used to protect the heart

sA can be used effectively to pro-
ıry, the degree of protection was
ed and to the conditions used for
nd A.P. Halestrap, unpublished
found to impair recovery of the
o be appropriate for use in open
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hat anti-oxidants and free-radical
ed heart from irreversible damage
sses within the cardiac myocyte
reagents, prevention of the mPT
that protection from reperfusion
mitochondrial calcium overload
ıse of calcium antagonists or
drial calcium uptake [71,89–97].
ıe mPT that such reagents would

, induced by the use of low extra-
rs of the Na^+/H^+ antiporter such
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ough low pH_i may have several
ofound inhibition of the mPT at
ıas been demonstrated directly in
ic/reperfused heart, mitochondr-
the pH_i takes to be restored from

an protect hearts [107–109] and
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ıve been attributed to beneficial
n free-radical production, since
9,110]. However, an additional
ı of the mPT, in part through its

free-radical scavenging effects. In addition, as a good respiratory substrate pyruvate might increase both the mitochondrial membrane potential, which would inhibit the mPT, and the NADH/NAD^+, which would help prevent oxidation of protein thiol groups critical for activation of the mPT [113–115]. Furthermore, pyruvate is transported into heart cells with a proton by means of the monocarboxylate transporter [65,116], which might lead to a decrease in pH_i directly. In parallel, competition between pyruvate and lactate for transport by the monocarboxylate transporter [65,116] may lead to a greater intracellular accumulation of lactic acid, further lowering of pH_i and thus causing additional inhibition of the mPT. We have demonstrated that the drop in perfusate pH of pyruvate-treated hearts on reperfusion is considerably greater than for control hearts. This implies that pyruvate causes a significant decrease in pH_i both at the end of ischaemia and during the reperfusion phase [87,117]. Indeed, there is also direct evidence from nuclear magnetic resonance studies that pyruvate causes a decrease in pH_i in a low-flow model of ischaemia [118]. Using the DOG technique we have confirmed that the protective effect of 10 mM pyruvate (present both before, during and after ischaemia) is accompanied by a reduction of mitochondrial pore opening during the initial stages of reperfusion and more extensive pore closure at later stages [87,119]. Thus, in the presence of pyruvate, hearts recover 100% of their LVDP after 40 min of ischaemia compared with only about 50% in the absence of pyruvate, and this is associated with DOG entrapment returning to pre-ischaemic values as opposed to only a 50% decrease in DOG entrapment in controls [87,117,119]. These data are the first direct evidence that mitochondrial pore opening can reverse fully when hearts recover their function during reperfusion.

Propofol

Propofol is an anaesthetic that is frequently used during cardiac surgery and in post-operative sedation [120]. There are reports that propofol can act as a free-radical scavenger [121,122] and also that, at concentrations higher than those used clinically, it may inhibit the PT of isolated mitochondria [123,124]. Other studies have indicated that propofol can attenuate the effects of reperfusion injury and hydrogen peroxide-induced oxidative stress of the perfused rat heart [125–127]. In our own studies we have confirmed that 2 μg/ml propofol, a concentration similar to that employed clinically, added 10 min prior to ischaemia and during reperfusion causes significant protection of isolated rat hearts from reperfusion injury (S. Javadov, P. Kerr and A. Halestrap, unpublished work). Recovery of LVDP after 30 min of ischaemia (means ± S.E.M.) increased from 36 ± 8% ($n = 10$) in control hearts to 70 ± 11% ($n = 8$; $P<0.05$) in propofol-treated hearts. This was accompanied by a 25% decrease in mitochondrial DOG entrapment. However, when added to isolated heart mitochondria at the same concentration, propofol afforded no protection against the mPT, suggesting that its protective effect against reperfusion injury may not be through a direct effect on the mPT. Rather, inhibition of the mPT may be secondary to propofol's reported abilities to lower cytosolic $[Ca^{2+}]$ through inhibitory effects on calcium channels [128,129] and to act as a free-radical scavenger [121,122]. Nevertheless, propofol provides another example

of a reagent whose protection of the heart from reperfusion injury is accompanied by a decrease in mitochondrial pore opening *in vivo*.

Preconditioning

Hearts subjected to two or three brief (3–5 min) ischaemic periods with intervening recovery periods before a prolonged period of ischaemia experience substantial protection against reperfusion injury [130,131]. It is thought that a major player in the mechanism of preconditioning is receptor-mediated activation of protein kinase C that occurs in response to the release of mediators, such as adenosine, bradykinin, endothelin-1, opioids and catecholamines, released during the brief ischaemic periods [130–134]. The means by which protein kinase C exerts its protective effects is not firmly established but a role for activation of K_{ATP} channels has been suggested since preconditioning is prevented by sulphonylureas, which are potent inhibitors of the K_{ATP} channel [135–137]. Whatever the mechanism of preconditioning, our own experiments using the DOG entrapment technique did not detect any decrease in mitochondrial pore opening (P.M. Kerr and A.P. Halestrap, unpublished work), although others have reported that mitochondrial damage is reduced in preconditioned hearts [138]. Thus protection of hearts and their mitochondria can occur without inhibition of the mPT. One observation that might account for how this is achieved is that the mitochondrial ATPase inhibitor protein is activated during the brief ischaemic periods [139,140]. The resulting inhibition of the ATPase may prevent 'open' mitochondria from degrading the ATP generated by glycolysis and the remaining 'closed' mitochondria. As a result, hearts would remain protected from reperfusion injury even when a significant number of mitochondria are in an 'open' state.

The mPT and apoptosis in the heart

Some myocytes in the failing heart undergo apoptotic cell death, as do myocytes in areas surrounding a myocardial infarct, i.e. areas that experience a less-pronounced ischaemic insult than that which leads to necrosis [141–147]. Recent evidence suggests that the mPT may act as the 'central executioner' of cells subjected to a range of insults, such as oxidative stress, growth factor removal or exposure to cytokines. Indeed, the mitochondria may not only determine whether a cell lives or dies, but also whether death occurs by apoptosis or necrosis [52,148–150]. Thus in some cells changes in $\Delta\Psi_m$ occur during early stages of apoptosis and can be inhibited by CsA, which also inhibits apoptosis [151,152]. Furthermore, mitochondria are required to induce apoptosis in a cell-free system, which they do by releasing apoptosis-inducing factors, the best characterized of which is cytochrome *c* [150,153–155]. In fact, work in this laboratory established many years ago that cytochrome *c* release does occur during the mPT [156]. The anti-apoptotic gene product, Bcl-2, is associated with the mitochondrial outer membrane and has been reported to inhibit the mPT and prevent release of cytochrome *c* and consequent caspase activation [157–159]. Attractive though this hypothesis may be, in many situa-

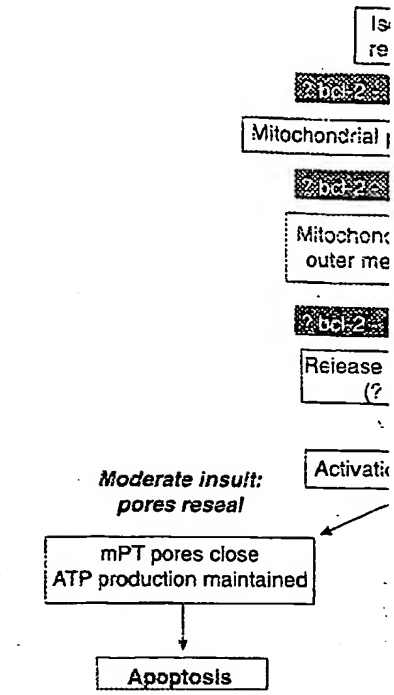

Figure 3 Scheme illustrating
deciding whether a cell dies by
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tions apoptosis can occur without e
does CsA protect every cell type fr
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seem likely that cells experiencing on
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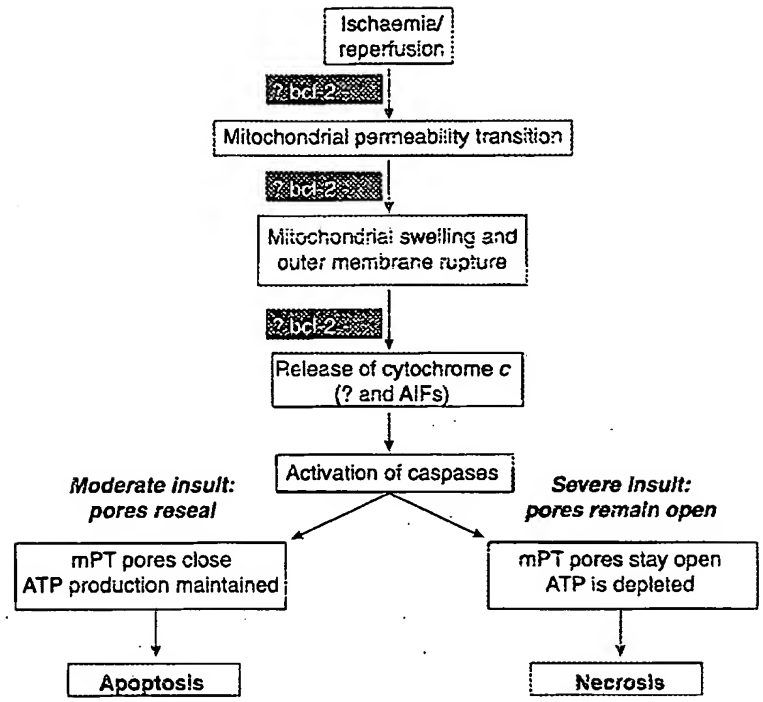

Figure 3 Scheme illustrating how the mPT may be involved in deciding whether a cell dies by necrosis or apoptosis. AIF, apoptosis-inducing factor.

tions apoptosis can occur without early changes in $\Delta\Psi_m$ [157,160–164]. Nor does CsA protect every cell type from all apoptotic stimuli, and it may even induce apoptosis under some circumstances [160,164–167]. However, it does seem likely that cells experiencing only a modest insult may undergo a transient opening of the mPT pore which then closes again, enabling ion gradients and ATP production to be re-established. Under such conditions, sufficient swelling of the outer membrane may occur to release cytochrome *c* and set in motion the apoptotic cascade that causes an organized, non-inflammatory cell death by apoptosis that can be attenuated by CsA. We have demonstrated that a clear example of this is the apoptotic cell death that is seen in the hippocampus 24 h after a 30 min period of insulin-induced hypoglycaemia [82]. However, the controlled nature of apoptosis requires that tissue ATP content is maintained, and where this is not the case cell death becomes necrotic [168,169]. Such a situation appears to occur in irreversible reperfusion injury where mPT pore opening is both extensive and prolonged. Under these conditions cytochrome *c* will be released, but mitochondria remain uncoupled and unable to generate the ATP required for maintaining cellular ionic homoeostasis. Neither can tissue damage be repaired and it will continue unabated, ultimately leading to rupture of the plasma membrane and cell death. This uncontrolled necrotic form of cell death is inflammatory and further exacerbated as neutrophil invasion leads to yet more damage. Thus the decision

between apoptosis and necrosis may rest on the extent of the mPT and can account for the observation that apoptosis and necrosis both occur in the reperfused heart, with the least damaged areas showing a preponderance of apoptosis over necrosis. A diagram summarizing how the mPT may act as the decision maker between apoptosis and necrosis is given in Figure 3.

Conclusions

The mPT converts the mitochondrion from an organelle that, through its production of ATP, sustains the cell in its normal function to an instrument of death. There is now strong evidence that the mPT involves a calcium-mediated conformational change in the ANT that converts it into a non-specific pore (see Figure 1). Except at very high [Ca^{2+}], mitochondrial CyP is required to implement this conformational change and the process is further sensitized to [Ca^{2+}] by oxidative stress. In contrast, decreasing pH_i below 7.0 greatly desensitizes the mPT to [Ca^{2+}]. This information can be used to devise strategies that may protect tissues such as the heart and brain from damage caused by ischaemia and reperfusion injury and thus has important clinical implications.

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